



Evaluation of a Novel Inaba Cholera Conjugate Vaccine in Mice

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Introduction

Vibrio cholerae is a noninvasive enteric pathogen and is responsible for cholera, a disease notable for severe diarrhea, rapid dehydration, and sometimes death if not adequately treated. Even though almost 150 years have passed since this pathogen and its major routes of transmission were documented (Koch 1884), cholera continues to exert a considerable burden in both endemic and epidemic settings.

Humans are the only known reservoir of *Vibrio cholera*, with a long and complex interaction between the pathogen and its host. Cholera has had a profound and sustained influence on human genetics and behavior. The enormous dread of cholera catalyzed the development of sanitation in 1800's, intravenous fluids, oral rehydration therapy, quarantines and further understanding of physiology and disease transmission that has reduced mortality not only of cholera but of many human diseases. Despite this progress, estimates suggest 2.8 million cases and 91,000 deaths due to cholera per year in endemic countries (Ali 2012).

Cholera was successfully eliminated from the Western Hemisphere in the 1887 through a combination of aggressive quarantine of incoming ships and sanitation improvement. Unfortunately, cholera was reintroduced in Peru in 1991 (Guerrant 2003). The bacteria inexorably marched back through previously cleared areas. A combination of denser populations, greater ease of transport, and weaker centralized power made duplicating the success of the previous century impossible. In Trujillo, Peru the attack rate in two months in 1991 was 2.6%. Due to contamination of the water supply and irrigation of produce field with sewage contaminated water, a large proportion of the population was exposed. A swift response of a public health system with adequate resources, as well as a knowledge of oral rehydration solution in the general population kept the case fatality rate at .04% (Swerdlow 1992). By contrast, in Haiti not only was the attack rate higher at 3.8%, but the case fatality rate was 1.4% (CDC 2012). The difference stems from weaker infrastructure or a more susceptible population, either from malnutrition or coinfections.

Epidemics such as the outbreak in Haiti demonstrate that delays in implementing the standard interventions of treatment centers, fluid support, and antibiotics can cost many lives (JAMA 2011). Additionally, the emergence of antibiotic resistant strains will continue to complicate case management (Faruque 2006). Unfortunately, both endemic and epidemic cholera continue to affect communities with challenges implementing cholera preventive or therapeutic measures. In such situations, an effective

cholera vaccine administered as a part of routine childhood immunizations or an immunization campaign could cheaply and effectively reduce the burden of cholera. A vaccine could theoretically protect from disease either by an antibacterial response preventing the replication of the bacteria within the small intestine or by an antitoxic response neutralizing the toxin without necessarily preventing replication of the bacteria. Cholera toxin is A-B heat labile enterotoxin, closely related to the labile toxin found in some strains of *E. coli* that also cause massive watery diarrhea. Five B subunits bind the ganglioside GM1 receptor on the surface of enterocytes and form a pore in the membrane of the enterocyte. The A protein enters the pore and activates intracellular adenylyl cyclase. The resulting increased level of cAMP stimulate the efflux of chloride ions through the CFTR channel. Water follows osmotically, resulting in large fluid losses (Mekalanos 1983).

Although an antitoxic response would be very effective at reducing morbidity and mortality in those vaccinated by interfering with the process outlined above, the advantage of an antibacterial response is that the person vaccinated is not only protected from clinical disease but also prevented from exposing others to a potentially deadly pathogen. Developing an effective cholera vaccine should be achievable because natural infection induces a sustained antibacterial and antitoxin response. Volunteers experimentally infected with cholera then challenged after three years showed complete protection against disease, which affected 4/5 naïve controls, and 3/4 previously infected volunteers did not shed any bacteria in their stool whereas all naïve volunteers shed bacteria in their stool (Levine 1981).

Koch recognized that individuals who recovered from cholera were protected from further disease in the same outbreak, but that they remained susceptible to outbreaks occurring in subsequent years. He used the acquirement of immunity to explain why cholera outbreaks resolved in places outside of India (Koch 1884). In endemic settings, much of the population carries a degree of immunity from past infection or exposure, and children bear the burden of severe disease and death. Although the protective efficacy of natural infection is the same in cases younger or older than 5 years (Ali 2011), current oral killed vaccines have demonstrated decreased efficacy and duration of protection in younger children. In particular, children have a decreased cellular (Leung 2012) and antibody (Leung 2013) response to vaccination, whereas this deficit is not seen in natural infection (Arifuzzaman 2012).

The major pathogenic serogroups of *V. cholerae*, O1 and O139, and major O1 serotypes, Inaba and Ogawa are defined by the arrangement of sugars in the O-specific polysaccharide (OSP) moiety in the membrane lipopolysaccharide LPS. Natural infection with *V. cholerae* Inaba induces protective immunity to reinfection with either Inaba or Ogawa strains, whereas infection with Ogawa protects against

reinfection with Ogawa but less well against Inaba (Ali 2011). The dominant serotype can shift as a critical proportion of the population develops protective immune responses against the endemic strains.

Experimental infection in immunologically naïve volunteers also shows profound and sustained protection that lasts at least three years after initial infection (Levine 1981) and to 6-10 years in epidemiologic studies (Koelle 2005). Currently available oral killed cholera vaccines have declining efficacy after 6-60 months (WHO 2010). Two licensed cholera vaccines, Dukoral and Shanchol, are currently in use, and more have been used in the past or started down the path of development (Shin 2011). No cholera vaccine, however, has demonstrated protective efficacy equivalent to that of natural exposure. Both Dukoral and Shanchol oral killed vaccines given in two doses and recommended oral booster every two years (WHO 2010)(Shin 2011). Dukoral has been shown to produce an overall memory B-cell response of lower magnitude and duration than natural cholera infection. Intriguingly, oral killed vaccines do not reproduce the robust memory B cell response to serospecific LPS observed to correlate with long-term protection following natural infection (Harris 2009). The memory response to cholera toxin and OSP is correlated with the affinity of the antibodies stimulated in the initial exposure (Alam 2013).

Specific mutants of LPS show that the OSP moiety is necessary and sufficient for the full passive immune effect of antibodies raised against LPS (Leitner 2013). The fact that there is no cross protection between serogroups of *V. cholerae* (Ali 2011) provides further evidence that immunologic response to the appropriate OSP moiety is necessary for protection. Since the Lipid A portion is responsible for the toxic effects of LPS, a vaccine composed of just the OSP could provide the beneficial effects without the toxicity. LPS preparations can also show a great variety in composition depending upon the manufacturing process (Johnson 2012). Any commercial vaccine preparation would require a pure and replicable preparation process.

OSP is entirely polysaccharide chains, which do not stimulate a robust immune response, especially among young children (Peltola 1984). Previous vaccines linking Inaba OSP to a protein have been tested in humans (Gupta 1998). Although multiple methods exist to conjugate protein to the OSP, the conjugation can alter the OSP structure, impeding immunogenicity (Xu 2011). A novel method to conjugate OSP to protein to increase immunogenicity without affecting the hapten structure was previously described (Xu 2011). Aluminum adjuvants enhance antibody response to vaccines and are widely used in currently licensed vaccines (HogenEsch 2002). Aluminum adjuvants have been specifically observed to increase the IgG response to conjugate polysaccharide vaccines (Wuorimaa 2001). Here, we

report characterization of an Inaba OSP cholera conjugate vaccine in mice administered with and without an aluminum adjuvant, with the aim to answer whether the OSP Inaba conjugate vaccine is immunogenic in mice and whether the addition of alum enhances the antibody response.

Methods:

We isolated Inaba LPS from solid colonies of *Vibrio cholera* O1 Inaba El Tor strain PIC018, a clinical isolate recovered in Bangladesh, via hot phenol extraction as previously described (Westfal 1965). The isolate was treated with enzymes (nuclease and protease), dialyzed against water and processed as has been previously described (Xu 2011). Briefly, we hydrolyzed the LPS with acetic acid to form OSP, which we extracted with equal volumes of chloroform, pelleted with ultracentrifugation, then lyophilized. We conjugated the OSP to BSA or a recombinant fragment of tetanus toxoid heavy chain (rTTHc), as previously described (Xu 2011, Alam 2014). To enhance purity, we introduced additional filtration steps of OSP to remove any product smaller than 3kDa, or larger than 30 kDa prior to use in conjugation.

Female Swiss-Webster mice three to five weeks old were intramuscularly vaccinated on days 0, 14, and 28 in cohorts of ten to fifteen mice. The vaccine was composed of Inaba OSP conjugated to rTTHc at a 5:1 molar ratio of OSP to rTTHc. Each injection consisted of 10 µg of OSP. In one group of mice, the vaccine was mixed in a 1:1 v/v ratio with Alum and delivered in a single injection. Control mice were vaccinated with Alum alone. We tail bled the mice on days 0, 7, 14, 28, 35, and 42. We centrifuged the blood at 5K g for ten minutes to isolate the serum, which we removed and stored at -80°C until processing.

Elisa:

We coated 96 well plates overnight at room temperature with either Inaba LPS (2.5 µg/mL in PBS, 100µl per well) or OSP-BSA. After a wash with PBS, we blocked the plates with 200 µl/ well 1% bovine serum albumin in phosphate buffered serum for forty minutes at 37°C to reduce non-specific protein binding. We diluted the serum 1:25 in 0.1% BSA-PBS-Tween (0.05%) and incubated the ELISA plate for ninety minutes at 37°C. After washing the plates three times with PBS-Tween .05%, we added 100 µl /well horseradish peroxidase conjugated goat anti-mouse IgG and IgM (Southern Biotechnology); 1:1000 dilution in .1% BSA-PBS-Tween (0.05%) and incubated for ninety minutes at 37°C. The plates were washed three times with PBS-Tween and once with PBS and read kinetically for five minutes at thirty

second intervals at an optical density at 405nm after the addition of 100 μ l per well of 12 μ l 30% H₂O₂ in 12 mL ABTS. The maximum slope for an optical density change of 0.2U was termed the milli-optical density units per minute (Alam 2014). Elisa units (EU) were calculated as the ratio to control pooled serum from mice in prior experiments.

Vibriocidal:

The vibriocidal assay has long been observed to be a correlate of protection against cholera, and has been characterized to largely correlate with IgM levels against LPS and OSP (Johnson 2012). We grew the vaccine strain of Inaba *V. cholera* O1 PIC018 overnight in 5 ml liquid culture (5ml LB with 5 μ l streptomycin). We made 4 1-mL aliquots of the liquid culture and spun at 5000 rotations per minute for ten minutes. We resuspended the pellet in 1 ml of 0.15 M saline and diluted to a standard concentration of 0.1 OD at 600nm. We heated 13 μ l serum for one hour at 56° C to inactivate the endogenous mouse complement. The serum was diluted 1:25 by adding 312 μ l sterile saline to the 13 μ l heat inactivated serum. Then we serially diluted the serum solution in duplicate at a 1:2 concentration. We added 39 μ l of the bacterial suspension and 11 μ l Guinea Pig Complement (Gibco BRL) to each well of the serially diluted serum samples and incubated sixty minutes at 37°C on a rocker for mild agitation. We added 150 μ l of brain heart infusion media to all wells and incubated at 37°C until the control wells without any complement were between 0.2 and 0.3 OD. We counted the vibriocidal response as the reciprocal of the highest dilution at which the OD of the well was less than half that of the control wells without serum.

Protection:

As previously described, we mixed wild type *V. cholera* with pooled serum from adult immunized mice (Alam 2014). In the challenge, we used cohorts of 20 3-5 day old unimmunized CD1 suckling mice. The pups were separated from their mothers for three hours, then we introduced 50 μ l of the mixture of bacteria and serum via a tube into the stomachs of infant mice. Deaths during the first 12 hours were considered to be related to the procedure rather than the bacteria. After the first 12 hours, the survival of the infant mice were counted every three hours. Animals surviving past 36 hours were euthanized.

Statistics and Graphs:

Groups were compared to each other using the Mann-Whitney U test. Within each group, we assessed the change from baseline using Wilcoxon Signed-Rank tests. We used Kaplan-Meier survival analysis to look at the protection in mouse pups. Statistical significance was based on two-tailed p-value less than

0.05. Graphs and calculations were produced using the GraphPad Prism 4 (GraphPad Software, Inc., La Jolla, CA).

Results

ELISA: By day 21, mice vaccinated with the conjugate vaccine developed a significant IgG response to OSP, whether or not adjuvant was used (Figure 1). The antibody response remained significantly elevated above baseline for the duration of the experiment. Mice vaccinated with the vaccine and adjuvant produced a significant IgG response to LPS by day 14, and mice vaccinated without the adjuvant produced a significant IgG response to LPS by day 35. Mice who received the vaccine with adjuvant did not produce a significant IgM response to either LPS or OSP. Mice receiving the vaccine alone produced a significant IgM response to OSP by day 14 and IgM response to LPS by day 21.

Vibriocidal: Both the mice vaccinated with the vaccine as well as the vaccine with Alum adjuvant produced a statistically significant increase in vibriocidal titer from baseline (Figure 2). Mice immunized with adjuvant alone did not produce a significant vibriocidal response.

Protection: The serum from mice vaccinated with the vaccine with Alum as an adjuvant demonstrated statistically significant protection compared with serum from mice vaccinated with Alum alone (Figure 3). Of the mice pups treated with serum from vaccinated mice, 10/17 (58.8%) survived to 36 hours compared with 1/13 (7.7%) ($p=.0006$) of mice who received serum from mice vaccinated with Alum alone.

Discussion

We demonstrated that a novel *Inaba* cholera conjugate vaccine induces a robust serologic response that inhibited growth of *V. cholerae in vitro* and also provided protection in the neonatal mouse challenge model. Notably, the addition of Alum as an adjuvant did not significantly improve response.

This vaccine consists of an OSP :rTThc conjugate. OSP alone is a T-cell independent antigen. Conjugating the carbohydrate OSP to a protein, even a protein not naturally encountered in conjunction with the carbohydrate, makes the conjugate accessible to the T-cell dependent pathway. The protein portion of

the conjugate can be displayed on antigen-presenting cells via MHC Class II molecules, particularly in germinal centers, where antigen-presenting cells interact with T-cells and B-cells. T-cells activated by the peptide-MHC are able to stimulate the B-cells that recognize the carbohydrate moiety not only to undergo affinity maturation and class switching but also to develop a subset of memory B-cells (Clarke 2013). Memory B-cells account for the ability to mount a rapid and specific antibody response to pathogens even decades after the initial exposure. Immunity generated by T-cell independent antigens wanes much more quickly, likely reflecting the shorter lifespan of plasma cells compared to memory B-cells. Adults with mature immune systems and primed B-cells are capable of mounting a boosted response to T-cell independent antigens. Children, particularly those less than two years of age, have a limited ability to mount an immune response to T-cell independent antigens (Clarke 2013). The failure of current oral killed vaccines to stimulate a memory B-cell response or maintain protection equivalent to natural infection (Harris 2009), along with the impaired protection in young children suggests that the current vaccines are not stimulating T-cell dependent immunity. Indeed, a memory T-cell response to cholera toxin in people of all ages receiving the oral cholera vaccine has been seen to predict later memory B cell responses to the same antigen. Though even young children are able to mount a memory T-cell response to natural cholera infection, they show impaired memory T-cell responses to oral cholera vaccines (Arifuzzaman 2012).

Tetanus toxoid presents an attractive conjugate protein, being safe and highly immunogenic in humans. Tetanus toxoid conjugate vaccines have FDA approval and are currently in commercial use. The use of tetanus toxoid in one version of the *Haemophilus influenzae* b conjugate vaccine illustrates the power of protein conjugation when targeting a polysaccharide from a pathogen with a large mortality and morbidity in infants and young children. Since evidence suggests that immune responses to subsequent reexposure to a polysaccharide in the absence of a stimulating peptide can be reduced over time (Clarke 2013), conjugating OSP to a protein might stimulate a memory response that can be strongly boosted upon exposure to natural infection or revaccination.

Cholera is an enteric pathogen and exposure and protection are mediated through interactions in the enteric mucosa. Further work is needed to assess the effect of this vaccine on protection at mucosal surfaces. Ultimately oral challenge studies in human volunteers would provide the clearest evidence of mucosal protection, but measuring antigen specific serum and fecal IgA may provide a correlation with protection. B-cells gain the ability to produce IgA after undergoing class switching. Naïve B-cells can be stimulated by T-cell dependent antigens or T-cell independent antigens to undergo class switching and

produce secretory IgA. Mucosal dendritic cells produce ligands such as APRIL and retinoic acid that stimulate class switching in antigen-specific B-cells (Gloudemans 2013). Both B and T cells home to the gut via upregulated expression of $\alpha 4\beta 7$ and CCR9. The expression of these receptors is induced by all-trans retinoic acid production of the antigen presenting cell, whether macrophages or dendritic cells. Small blood vessels in the gut constitutively express the ligands for these receptors CXCL13, which allows the binding and extravasation of the appropriately marked cells (Mora 2008). The use of Alum as an immunoadjuvant has been well documented. Our results demonstrate a predominant effect of Alum on the IgG response, specifically anti-OSP IgG, with a decreased IgM response in mice receiving the adjuvant. These results are consistent with the previously reported predominance effect of Alum on the IgG response. In early studies into the effect of Alum, the main most notable response was seen in the IgG production, specifically IgG1 (Fujimaki 1984). Although Alum is FDA approved as a vaccine adjuvant in humans, other adjuvants at earlier stages of development may hold promise for both augmenting the magnitude of response as well as directing the response towards mucosal immunity. Certain adjuvants including retinoic acid and cholera toxin itself have been shown to induce mucosal immune response when administered as a component in transcutaneous immunization (Martin 2010). The administration of retinoic acid with subcutaneous cholera toxin also produced a protective mucosal response (Hammerschmidt 2011). Future work can evaluate combination of the vaccine with other adjuvants such as retinoic acid or cholera toxin.

Antibodies against the OSP moiety limit the growth of *V. cholerae in vivo* and *in vitro* which is consistent with accessible location of the polysaccharides on the surface of the bacteria. The specificity of the OSP:rTTHc vaccine for generating antibodies against OSP as well as the production of a vibriocidal effect hold promise as either a primary vaccine or as a boost to be given in conjunction with oral vaccines. The antibodies that contribute to the vibriocidal response depend on the epitopes that stimulated the response. A high baseline vibriocidal titer correlates, albeit imperfectly, with protection among household contact of patients with cholera (Saha 2004). In this experiment, we used the same strain of *V. cholerae* used to produce the vaccine in the vibriocidal assay. The initial outbreak in Haiti was caused by an Ogawa serotype organism (Talkington 2011). Isolates of the same genetic strain expressing Inaba OSP have been now isolated, however, demonstrating the ability of the bacillus to change serotype (CDC 2012). Shifts in the predominant serotype of cholera can occur over time as a critical proportion of the population develops a protective antibody response (Koelle 2006). Future work can assess cross-protection against Ogawa, and also assess vaccination with a mixture of Ogawa and Inaba conjugate vaccines. Achieving broad protection will likely require the use of multiple strains in the production of a

vaccine, although an Inaba based vaccine would give the broadest protection from a monovalent immunization.

This study was limited by the use of a model animal system, which is nonetheless a necessary step prior to advancing trials to humans. Although the pathogenicity of *V. cholerae* is different in mice than humans and does not cause disease in adult mice, this study used mouse model to predominantly assess immunogenicity of the vaccine. Given the differences between mice and humans, our results would not a priori predict the exact response in humans, but are useful in qualitatively comparing the response to different vaccine formulations and adjuvants as they are developed.

Immune correlates of protection facilitate vaccine research, allowing for accelerated screening of candidate vaccines prior to large efficacy trials in humans. Although current immune correlates of protection for cholera are reliable, further elucidation of the immune responses to target both short and long-term immunity will help further efforts to develop a cholera vaccine stimulating lasting immunity.

In conclusion, cholera is a disease of poverty, persisting in places with limited access to sanitation facilities and safe water. The industrial revolution in Britain stimulated a massive urbanization, with poor families living in crowded conditions, often in areas with open sewage that emptied into the Thames, the main water source for those same people. Although most people living in London in the 19th century recognized that poor areas such as Soho carried the brunt of the successive cholera pandemics, the blame was placed on miasma- “bad air” or simply a moral shortcoming among the poor. John Snow famously identified a well on Broad Street as the source of infection for an outbreak.

John Snow, in publishing a pamphlet on the communication of cholera (Snow 1855), grasped the implications that understanding the epidemiology of the disease would have on efforts to control its spread:

“The belief in the communication of cholera is a much less dreary one than the reverse; for what is so dismal as the idea of some invisible agent pervading the atmosphere, and spreading over the world? IF the writer’s opinions be correct, cholera might be checked and kept at bay by simple measures that would not interfere with social or commercial intercourse; and the enemy would be shorn of his chief terrors. It would only be necessary for all persons attending or waiting on the patients to wash their hands carefully and frequently, never omitting to do so

before touching food, and for everybody to avoid drinking, or using for culinary purposes, water into which drains and sewers empty themselves; or, if that cannot be accomplished, to have the water filtered and well boiled before it is used.”

Once the waterborne nature of the disease was identified, efforts were made to emphasize clean sources of water in addition to general sanitation. Queen Victoria and Prince Albert introduced sanitation reforms based on massively improved public infrastructure of water and sewage that along with a general increase in standard of living removed the threat of cholera epidemics in London. Likewise, today cross-disciplinary development focusing on improved standards of living holds the final answer to the pathogenic potential of cholera. In the interim, OSP-conjugate vaccines hold promise to limiting the burden of cholera among all populations, including children, in endemic areas, as well as providing a swifter answer among epidemic stricken populations than that afforded by current oral killed cholera vaccines.

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ETR, MB and GE designed the experiment. MB, YY, and GE prepared the LPS and OSP. MB administered the vaccine. MB, GE and YY tail bled the mice. GE performed the ELISA and vibriocidal assays. MB, YY, and GE performed the survival assay in mice.

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Figures

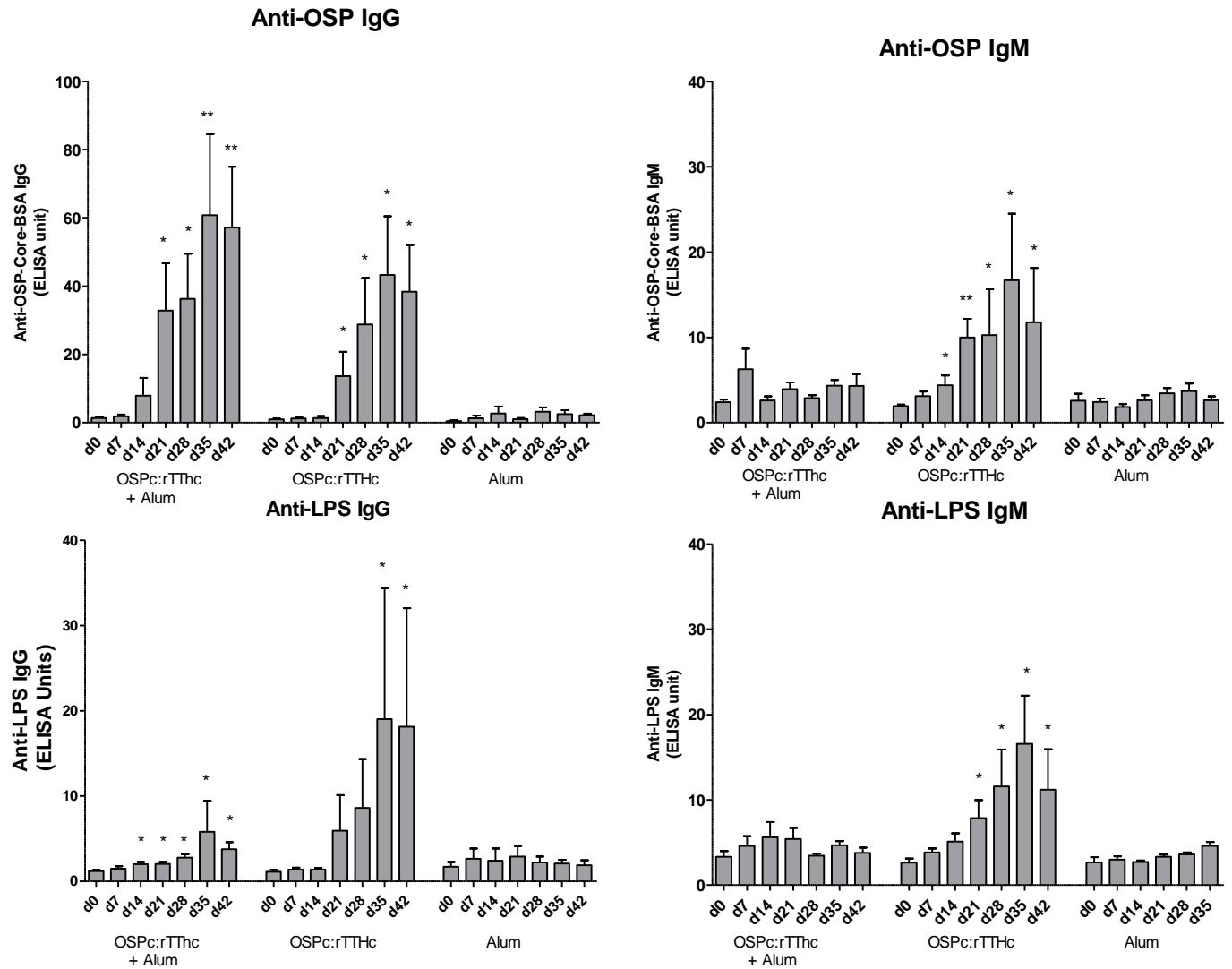


Figure 1. ELISA response in mice vaccinated with and without adjuvant.

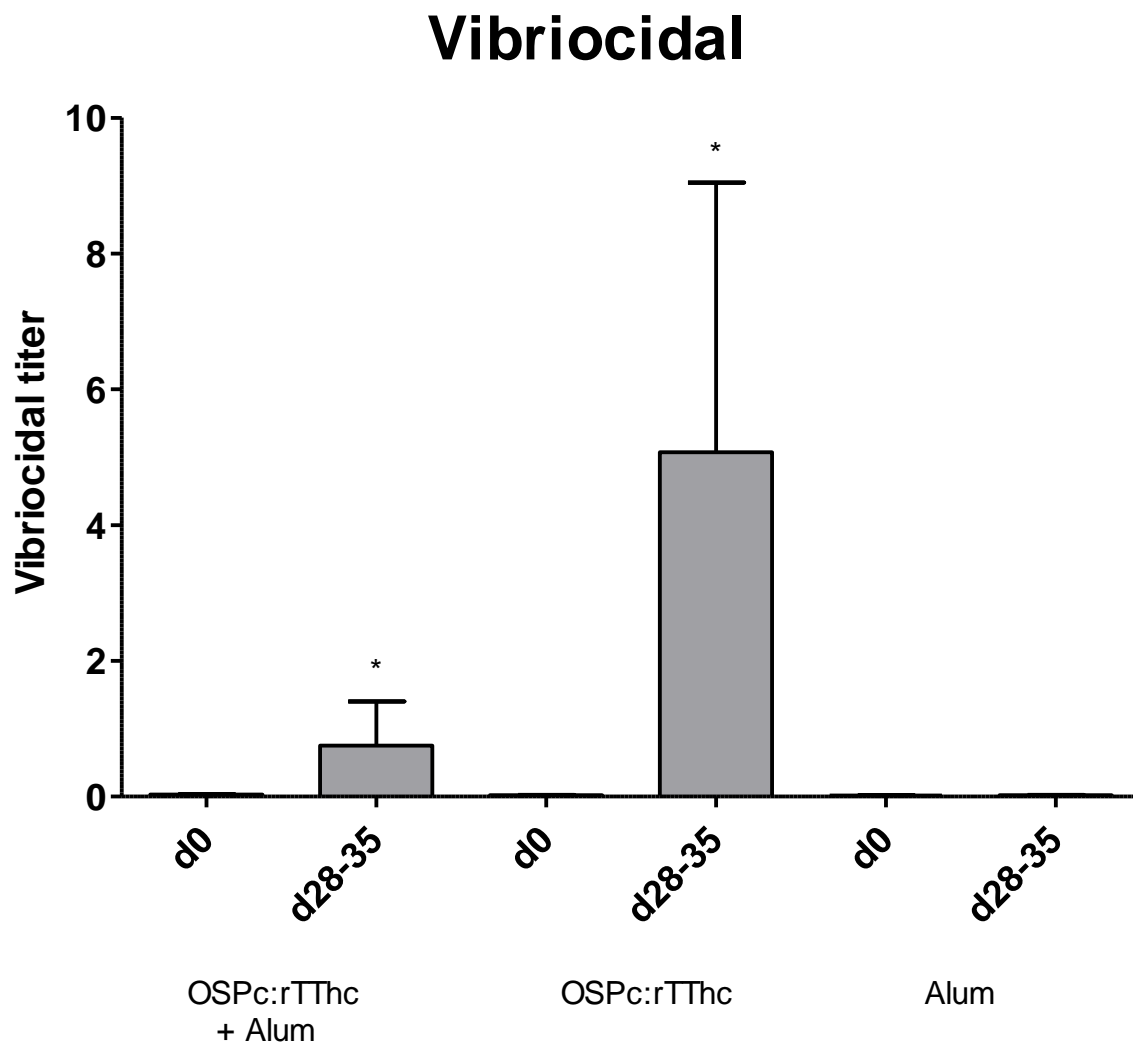


Figure 2. Vibriocidal titer. Both the conjugate vaccine alone and when combined with Alum produced a statistically significant response over baseline.

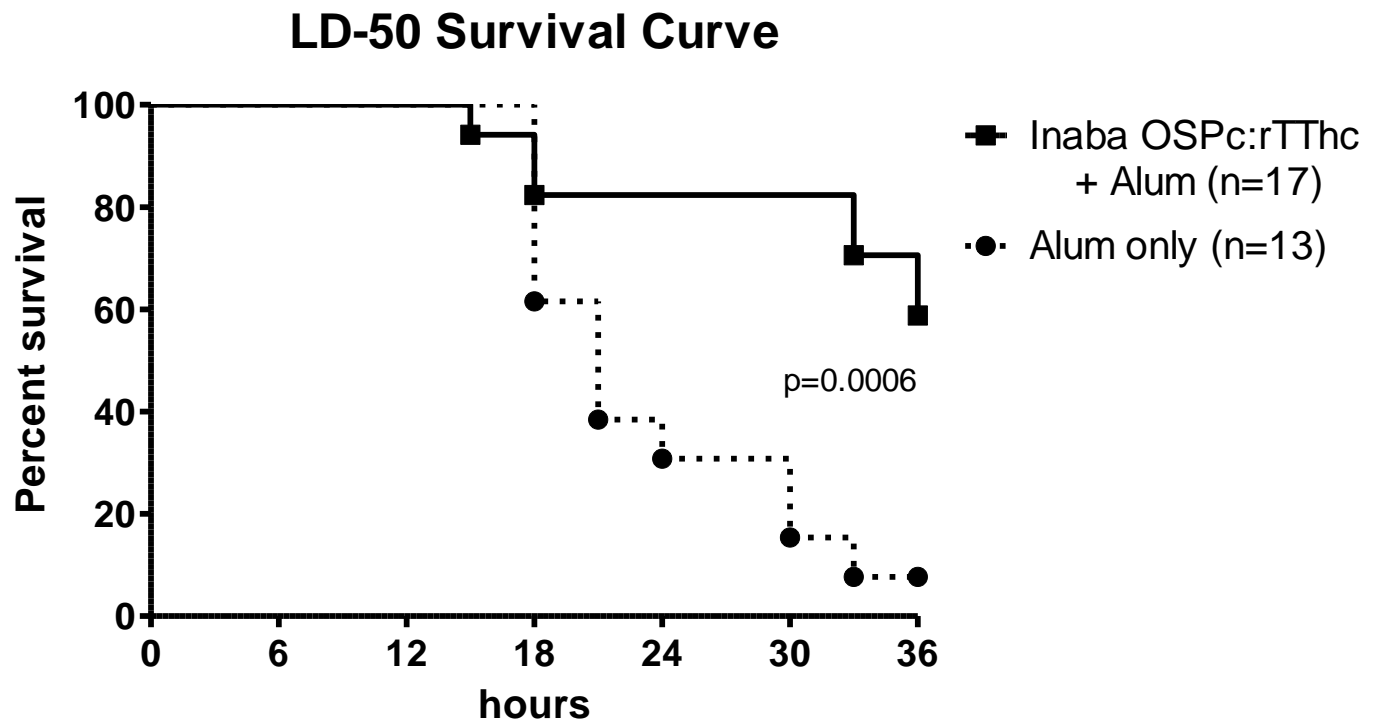


Figure 3. Protection assay in infant mice. Of the mice pups treated with serum from vaccinated mice, 10/17 (58.8%) survived to 36 hours compared with 1/13 (7.7%) ($p=0.0006$) of mice who received serum from mice vaccinated with Alum alone.